Int. J. Morphol.,
42(6):1679-1685, 2024.

Targeting the Dysregulation of Pancreatic AMPK-iNOS-p53 Axis by Metformin in a Rat Model of L-arginine-Induced Acute Pancreatitis

Tratamiento de la Desregulación del Eje AMPK-iNOS-p53 Pancreático Mediante Metformina en un Modelo de Rata de Pancreatitis Aguda Inducida por L-arginina

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ALZAMIL, N.M. & AL-HASHEM, F. Targeting the dysregulation of pancreatic AMPK-iNOS-p53 axis by metformin in a rat model of L-arginine-induced acute pancreatitis. *Int. J. Morphol., 42(6)*:1679-1685, 2024.

SUMMARY: A severe form of the inflammatory disease, acute pancreatitis, can induce multiple organ failure, leading to a high mortality rate. Little is known about the negative interaction between AMP-activated protein kinase (AMPK) and the nitrosative stress biomarker inducible nitric oxide synthase (iNOS), as well as the apoptosis biomarker tumour suppressor p53 in pathological conditions. Therefore, we tested the hypothesis that acute pancreatitis can cause the pancreatic AMPK-iNOS-p53 axis to become dysregulated, and the inducer of AMPK, the hypoglycemic, anti-inflammatory, and antioxidant drug metformin, can ameliorate the disease. In this study, acute pancreatitis was induced in rats using two injections of L-arginine (2.5 gm/kg) and culled after two days. The second group (protective group) was pretreated for two weeks with 50 mg/kg metformin prior to the induction of acute pancreatitis and kept on metformin until sacrificed with other groups. Pancreatic injuries developed in the model group (L-arginine) demonstrated by a substantial increase in iNOS and p53 immunostaining, as well as biomarkers of pancreatic injury, and AMPK inhibition. All these investigated signaling molecules were significantly ($p<0.001$) modulated by metformin. In addition, a significant correlation was detected between iNOS and AMPK, p53, as well as biomarkers of acute pancreatitis (amylase, lactate dehydrogenase, and myeloperoxidase). Thus, these findings demonstrate an association between acute pancreatitis and the modulation of the pancreatic AMPK-iNOS-p53 axis while being protected by metformin.

KEY WORDS: Acute pancreatitis; iNOS; p53; AMPK; Metformin, Rat model.

INTRODUCTION

The severe form of acute pancreatitis (AP) represents a significant problem or task to the healthcare system due to a very high mortality rate, which can reach around 45 % when systemic inflammation and multiple organ failure develop. This contrasts with a low mortality rate of around 2 % in mild acute pancreatitis (Popa *et al*., 2016). In the USA, AP is the leading cause of hospital admissions for patients with gastrointestinal disorders, with an estimated annual cost exceeding $2.6 billion (Anderson *et al*., 2023). Many factors can cause the pathogenesis of AP, such as common bile duct blockage, heavy alcohol consumption, blunt trauma to the upper abdomen, adverse drug reactions, infection, and sepsis (Wang *et al*., 2009).

Animal models of experimental AP are useful for carefully understanding the physiopathology / disordered physiological processes of the disease and testing possible drugs for therapeutic purposes (Kui *et al*., 2015). For example, intraperitoneal injections of 2.5 - 4 g/kg of the amino acid L-arginine induced AP in rodents like mice and rats (Czakó *et al*., 2000; Kui *et al*., 2015). L-arginine induces AP by two mechanisms: (i) the conversion of L-arginine to nitric oxide by the NOS enzyme and subsequently to peroxynitrite (a highly cytotoxic compound) when reacting with oxygen radicals, which cause tissue damage via nitrosative stress pathway (Venardos *et al*., 2009); and (ii) the hydrolysis of L-arginine by arginase to L-ornithine and

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FUNDING. This research was funded by Princess Nourah bint Abdulrahman University Researchers Supporting Project number (PNURSP2024R211), Princess Nourah bint Abdulrahman University, Riyadh, Saudi Arabia; and by the Research Deanship of King Khalid University, Abha, Saudi Arabia; Grant number No. GRP/119/45.

Received: 2024-06-09 Accepted: 2024-08-14

urea. A severe type of the disease (AP) is reported to be induced by L-ornithine in rats (Biczó *et al*., 2010). Additionally, other compounds such as cerulean, the equivalent of cholecystokinin hormone (Kim, 2008), and ethanol (Pandol *et al*., 2003) have been successfully used in animal models of AP. These compounds act via impairment of the production and secretion of the digestive enzyme, vacuolization of cytoplasm and necrosis of acinar cells, inflammatory pancreatitis, and formation of edema.

Metformin is a well-known pleotropic drug that induces the production of AMPK (Hawley *et al*., 2002), and AMPK activation inhibits iNOS in several cell types, like adipocytes, macrophages, and myocytes (Pilon *et al*., 2004). Additionally, nitrosative stress (iNOS) is located upstream of apoptosis (p53) (Sandau *et al*., 1997). Furthermore, metformin is reported to ameliorate the advancement of pancreatic cancer (Li *et al*., 2017), metabolic dysfunction-associated fatty liver disease (Matafome *et al*., 2011), hepatic injuries induced by thioacetamide (Al-Hashem *et al*., 2019), and apoptosis caused by stress in primary rat hepatocytes (Conde De La Rosa *et al*., 2015). Therefore, we guessed that modulation of the AMPK-iNOS-p53 axis in pancreatic tissue of rats with acute pancreatitis could be suppressed with metformin.

MATERIAL AND METHOD

Animals. The ethical committee at King Khalid University approved all experimental procedures in accordance with the Guide for the Care and Use of Laboratory Animals published by NIH publication No.85-23, revised 1996. Wistar male rats (150-200 g) were held in a clean place at room temperature (RT) and with a 12h light: 12 h dark cycle, and had ready access to food and water.

Experimental design. Following the adaptation period, 24 rats were assigned into 3 groups as follows: (i) control rats: received intraperitoneal injection of vehicle ; (ii) rats that were injected intraperitoneally after two weeks with L-arginine (2.5 mg/kg, 2 doses) at 1-hour intervals (L-arg group, the model group) (Czakó *et al*., 2000); and (iii) the third group of rats (Met +L-arg) treated with 50 mg/kg metformin for the first 14 days and injected with L-arginine on day 15. The rats continoued receiving metformin for another two days. At day 17, samples of blood were withdrawn under anesthesia, and rats were then culled using cervical dislocation and pancreatic tissues were harvested.

Assessment of blood levels of amylase, LDH, MPO, and TNF-α. Kits purchased from Abcam, Cambridge, UK were used to assess serum levels of amylase, lactate dehydrogenase (LDH), and TNF-α. Whereas, MPO was assessed by kits purchased from EagleBio, NH, USA. All kits were used according to the instructions of the manufactures.

AMPK western blotting analysis. 25 µg of extracted protein (pancreas tissues) per sample were immunoblotted as mentioned before (Alshahrani *et al*., 2024). Briefly, The membranes were incubated at 40 °C overnight with anti-AMPK-phospho-Thr172 purchased from Cell Signaling Technology, Beverly, MA, USA. Protein bands were visualized using ECL detection kit (Amersham-Pharmacia, UK). Image analysis software was used to obtain the intensity of the assessed protein after normalization by b-actin on the Chemi Doc MP imager.

Immunohistochemistry of iNOS, p53, and CD45. Pancreatic tissue sections prepared from the paraffin blocks were incubated with the primary antibodies; anti-iNOS, anti-p53, and anti-CD45 obtained from Abcam, Cambridge, UK for 15 h in a humidity chamber following the retrieval of antigen. Then, washed tissue sections were incubated with the secondary antibody for 30 min at RT. Sections were co-stained with Meyer hematoxylin.

Statistical analysis. The analyses of generated data were performed using version 10 of the SPSS. One-way ANOVA then Tukey's *post hoc* test was used to reach statistical comparisons of data. Pearson correlation was completed to find a probable significance between two different parameters. $p \leq 0.05$ was established to be statistically significant.

RESULTS

A Rat model of acute pancreatitis induces by L-arginine (L-arg). Acute pancreatitis (AP) was observed in rats 48 hours post-injection of L-arg, demonstrated by a substantial increase in the inflammatory biomarker TNF-α (Fig. 1A), infiltration of inflammatory cells into the pancreas measured by CD45 (leukocyte common antigen) expression (Figs. 1C and 1D), and an increase in the biomarker of pancreatitis, serum amylase (Fig. 1B). This enabled us to investigate the dysregulation of the pancreatic AMPK-iNOS-p53 axis.

Metformin protects the pancreatic AMPK-iNOS-p53 axis modulated by L-arginine (L-arg). In cell signaling, the iNOS-p53 axis is located downstream of AMPK (Pilon *et al*., 2004), and pancreatic tissue levels of AMPK decrease in an inflamed pancreas (Yang *et al*., 2020). We investigated the pancreatic AMPK-iNOS-p53 axis in acute pancreatitis with and without metformin. Injecting rats with two doses of L-arg caused a sharp increase in iNOS (Fig. 2) and p53 (Fig. 3) protein expression after 48 h, associated with the inhibition of the phosphorylated AMPK (Fig. 2A). Immunohistochemical staining of pancreatic tissue sections obtained from the

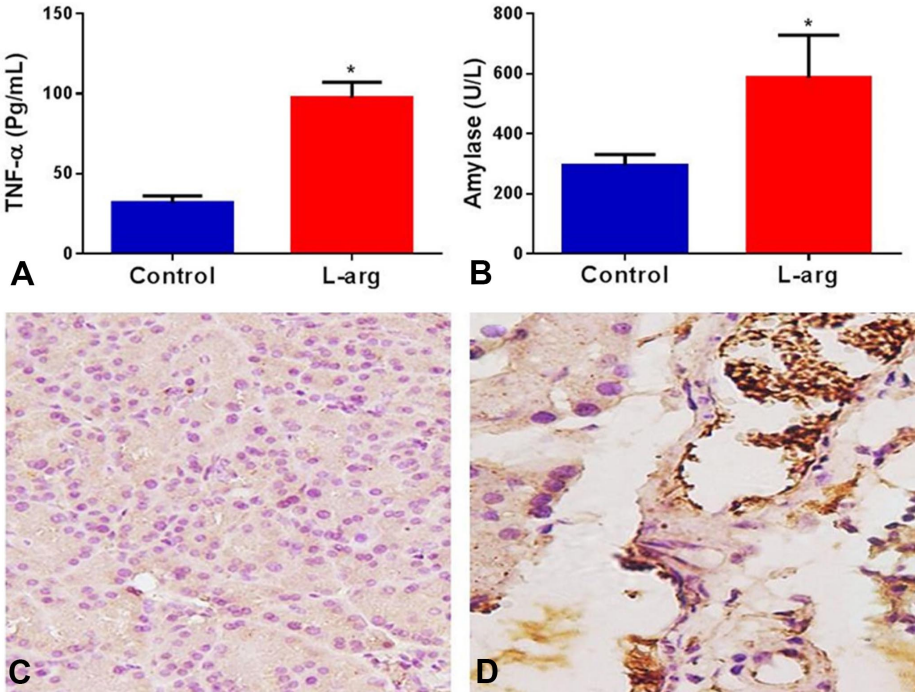

Fig. 1. L-arginine (L-arg) causes in rats AP. Blood levels of TNF-α (A) and amylase (B) were determined in the control and model (L-arg) rats' groups two days post injecting the model group with L-arg. All p values are significant. *p<0.0001 versus control. (C and D) Immunohistochemistry of CD45 (X200) of pancreas sections from the control (C) and L-arg (D) groups are shown. AP: acute pancreatitis; TNF-α: tumor necrosis factor-alpha; CD45: leukocyte common antigen.

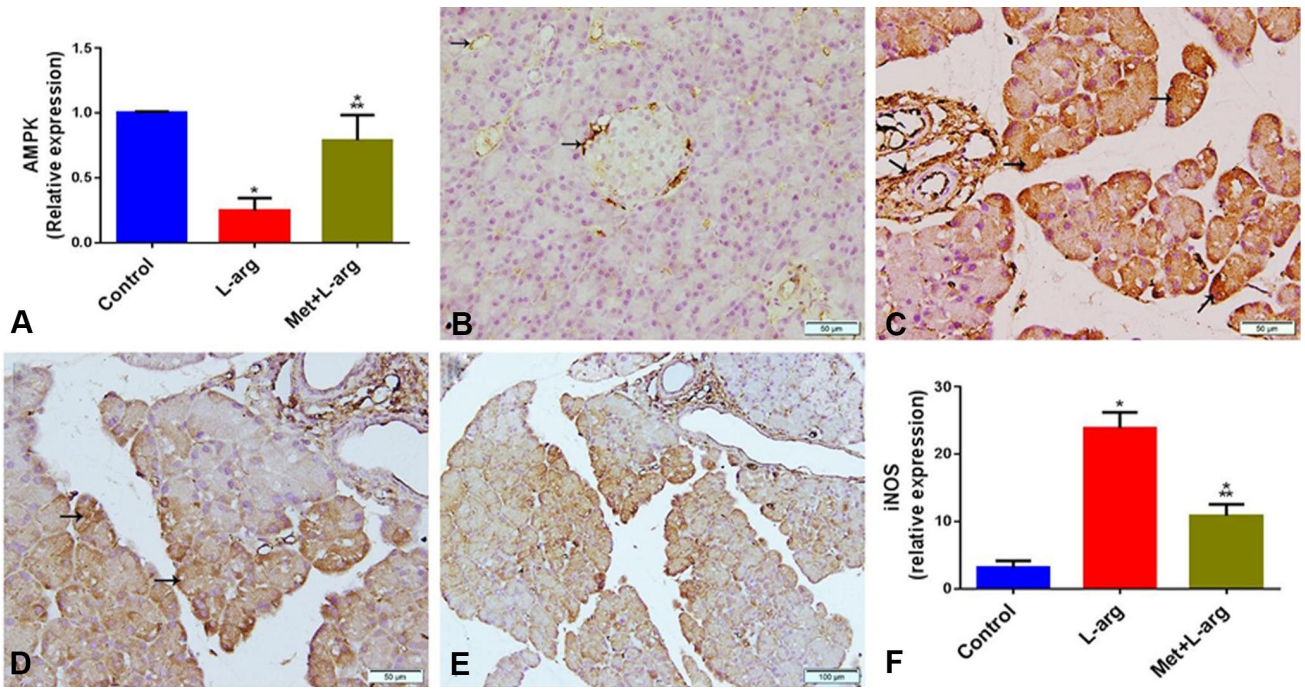

Fig. 2. Induction of the pancreatic AMPK-iNOS axis dysregulation by L-arginine (L-arg) is protected by metformin (Met). Levels of the active form of AMPK (p-AMPK) in all rats were assessed by Western blotting and the relative expression is displayed in (A). Immunohistochemistry of iNOS in pancreas sections (B, C, D, x200; E, x100) from the control (B), L-arg (C), and Met+L-arg (D and E) groups of rats are shown. Quantification of iNOS immunostaining area % from these groups is displayed (F). Arrows point to the positive iNOS-immunostained cells. All p values are significant. *p≤0.0245 versus control, **p<0.0001 *versus* L-arg. AMPK: AMP-activated protein kinase; iNOS: inducible nitric oxide synthase.

untreated L-arg group displayed a significant widespread of strong positive iNOS immunostaining in the parenchyma of the pancreas and in the blood vessel wall (Figs. 2C and 2F) compared to control rats that displayed negative to weak positive immunostaining (Fig. 2B). Metformin treatments (Figs. 2A and 2D-F) significantly ($p<0.001$) but not completely prevented the effects of L-arg on the pancreas as demonstrated by a scattered mild positive iNOS immunostaining.

P53 positive immunostaining was also observed in tissue sections prepared from the model group demonstrated by numerous immunostained-cells in the parenchyma of the pancreas and a strong P53 nuclear immune-reaction (Figs. 3B and 3E). Metformin treatments before and after L-arg injections (Figs. 3C-E) significantly ($p<0.001$) reduced p53 tissue expression demonstrated by mild to moderate p53 immune reaction but still significant ($p<0.001$) to control group (Figs. 3A and 3E).

Induction of biomarkers of acute pancreatitis by L-arginine (L-arg) is inhibited by metformin. We further assessed serum levels of biomarkers of pancreatic injuries (Table I) in these rats. These biomarkers are well-known indicators of the disease (Matull *et al.*, 2006). The results showed about a two-fold increase in amylase, LDH, MPO, and TNF-α levels, which were significantly ($p<0.001$) but partially inhibited by metformin treatment.

Correlation between iNOS score and pancreatic p53, AMPK, and biomarkers of acute pancreatitis. The link between the score of nitrosative stress (iNOS) and pancreatic apoptosis (p53), AMPK, and biomarkers of acute pancreatitis caused by L-arg is displayed in Figures 4A-F. A significant ($p<0.0001$) correlation was detected between iNOS and AMPK ($r = - 0.928$), p53 ($r = 0.970$), amylase ($r = 0.851$), LDH ($r = 0.887$), MPO ($r = 0.872$), and TNF-α ($r = 0.898$).

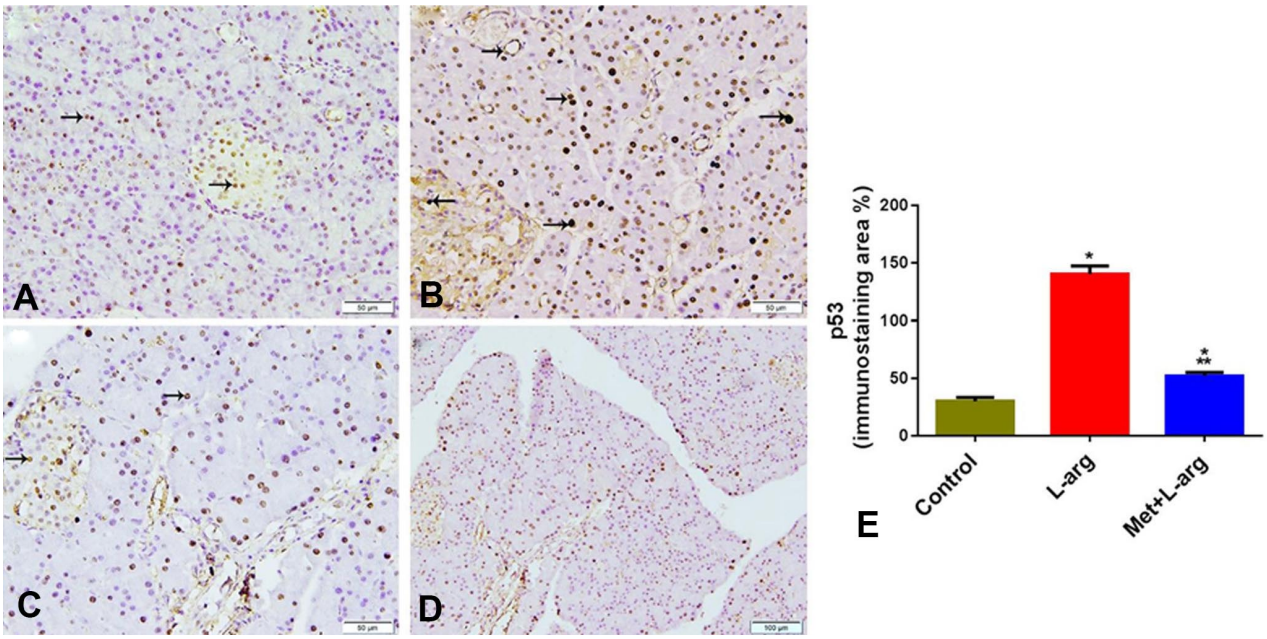

Fig. 3. Induction of the pancreatic P53 by L-arginine (L-arg) is protected by metformin (Met). Immunohistochemistry of p53 in pancreas sections (A,B,C, x200; D, x100) from the control (A), L-arg (B), and Met+L-arg (C and D) groups of rats are shown. Quantification of p53 immunostaining area % from these groups is displayed (E). Arrows point to the positive p53-immunostained cells. All p values are significant. *$p<0.0001$ versus control, **$p<0.0001$ *versus* L-arg. p53: tumour suppressor p53.

Table I. Effects of metformin on L-arg-induced biomarkers of pancreatic injury. Blood levels of amylase, MPO, LDH, and TNF-α were assessed in all rats 48 hours post L-arg injections.

	Control	L-arg	Met+L-arg
Amylase (U/L)	291.1±42.1	580.9±148.1*	429.7±17.2*#
MPO (U/mL)	0.79±0.16	2.20±0.39*	1.33±0.40*#
LDH (U/L)	121.2±8.4	279.7±40.9*	172.4±38.1*#
TNF-α (pg/mL)	31.1±4.5	95.2±9.50*	51.4±16.5*#

L-arg: L-arginine; Met: metformin; MPO: myloperoxidase; LDH: lactate dehydrogenase; TNF-α: tumor necrosis factor-alpha. Presented p values are all significant ($p<0.05$). *: versus control, # *versus* L-arg.

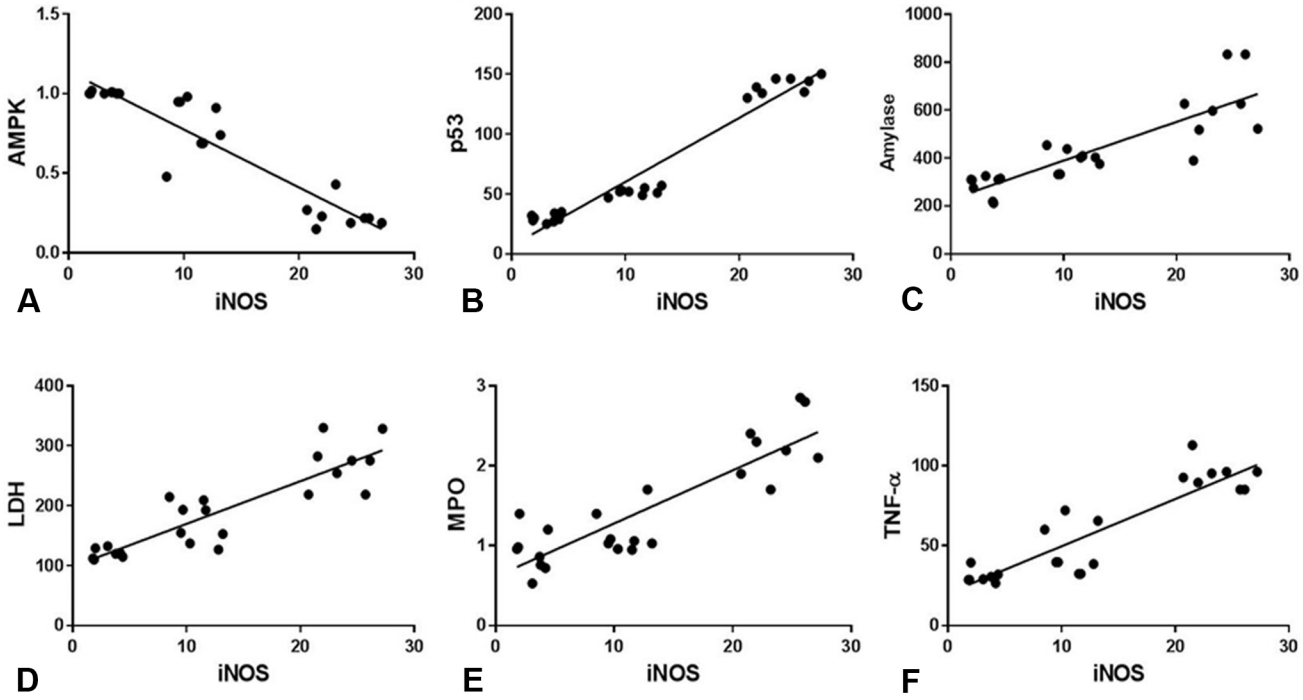

Fig. 4. Pancreatic iNOS score correlates with AMPK, apoptosis, and biomarkers of pancreatic injury. A significant ($p<0.0001$) link was noted between iNOS versus AMPK (A), p53 (B), amylase (C), LDH (D), MPO (E), and TNF-a (F). iNOS: inducible nitric oxide synthase; AMPK: AMP-activated protein kinase; p53: tumour suppressor p53; LDH: lactate dehydrogenase; MPO: myeloperoxidase; TNF-α: tumor necrosis factor-alpha.

DISCUSSION

These studies investigated the pancreatic AMPK-iNOS-p53 axis in an animal model of acute pancreatitis (AP) 48 hours post L-arginine (L-arg) injections with and without the hypoglycemic, antioxidant, and anti-inflammatory drug metformin. We also examined the potential association between these parameters and biomarkers of AP. Using immunohistochemistry, special histology staining methods, western blots, and blood chemistry, we demonstrated the dysregulation of the AMPK-iNOS-p53 axis triggered by L-arg, which appeared to be protected by metformin (Fig. 5). Furthermore, a significant link between nitrosative stress (iNOS) and the above-mentioned parameters, as well as AP biomarkers, was established, highlighting the protective effects of the pleiotropic drug metformin. Therefore, our results were undeviating from our working hypothesis that the induction of acute pancreatitis is associated with AMPK-iNOS-p53 axis dysregulation, which can be mitigated by metformin.

Autodigestion of the pancreatic tissue due to activation of digestive enzymes, such as trypsin in the pancreatic duct, leads to the inflammation of the pancreas (Ceppa *et al*., 2011). It has been described that acute pancreatic injury causes a substantial increase in the levels of inflammatory cytokines, such as TNF-α, and tissue necrosis biomarkers like amylase and LDH, linking these

Fig. 5. Proposed animal model for AP induced by L-arginine (L-arg) and protected by metformin (Met). AP: acute pancreatitis; AMPK: AMP-activated protein kinase; iNOS: inducible nitric oxide synthase; p53: tumour suppressor p53.

parameters to the pathology of the disease (Uhl *et al*., 1991; Wang *et al*., 2017). Additionally, the antioxidants N-acetylcysteine and resveratrol inhibited TNF-α production in AP caused by bile-pancreatic duct obstruction (de Dios *et al*., 2006) and necrotizing pancreatitis induced by L-arg (Wang *et al*., 2017), respectively. The level of the tissue-protective enzyme AMPK is severely decreased in patients with pancreatic cancer and AP (Chen *et al*., 2017; Yang *et al*., 2020). Furthermore, (i) iNOS and mutated p53 expression were increased in a mouse model of pancreatic cancer (Chattopadhyay *et al*., 2020); (ii) chronic ulcerative colitis is associated with the augmentation of TNF-α, iNOS, and p53 (Goretsky *et al*., 2012); (iii) transfection of human and rat vascular smooth muscle cells with iNOS cDNA induced apoptosis (increased p53 gene expression) (Iwashina *et al*., 1998); and (iv) a systematic review and meta-analysis suggested that metformin is the best antidiabetic drug to prevent pancreatic cancer in type 2 diabetes mellitus patients (Wang *et al*., 2014). These reports corroborate our findings of pancreatic AMPK-iNOS-p53 axis dysregulation in AP, while also showing it can be protected by metformin.

ACKNOWLEDGMENTS. The author would like to thank Professor Bahjat Al-Ani from the Department of Physiology, College of Medicine, King Khalid University, Abha, Saudi Arabia for his input and help during the preparation of this manuscript. We also thank Dr. Mariam Al-Ani from Face Studio Clinic, 90 Hagley Road, Edgbaston, Birmingham, B16 8LU, UK for proofreading the manuscript.

ALZAMIL, N.M. & AL-HASHEM, F. Tratamiento de la desregulación del eje AMPK-iNOS-p53 pancreático mediante metformina en un modelo de rata de pancreatitis aguda inducida por L-arginina. *Int. J. Morphol., 42(6)*:1679-1685, 2024.

RESUMEN: Una grave forma de enfermedad inflamatoria, la pancreatitis aguda, puede inducir insuficiencia orgánica múltiple lo que conduce a una alta tasa de mortalidad. Existe poca información de la interacción negativa entre la proteína quinasa activada por AMP (AMPK) y el biomarcador de estrés nitrosativo óxido nítrico sintasa inducible (iNOS), así como el biomarcador de apoptosis supresor de tumores p53 en condiciones patológicas. Por lo tanto, probamos la hipótesis de que la pancreatitis aguda puede causar que el eje pancreático AMPK-iNOS-p53 se desregule, y el inductor de AMPK, el fármaco hipoglucemiante, antiinflamatorio y antioxidante metformina puede mejorar la enfermedad. En este estudio, se indujo pancreatitis aguda en ratas usando dos inyecciones de L-arginina (2,5 g/kg); las ratas fueron sacrificadas después de dos días. El segundo grupo (grupo protector) fue tratado previamente durante dos semanas con 50 mg/kg de metformina antes de la inducción de pancreatitis aguda y se mantuvo con metformina hasta que se sacrificaron con otros grupos. Las lesiones pancreáticas desarrolladas en el grupo modelo (L-arginina) se demostraron por un aumento sustancial en la inmunotinción de iNOS y p53, así como en los biomarcadores de lesión pancreática y la inhibición de AMPK. Todas estas moléculas de señalización investigadas fueron moduladas significativamente ($p<0,001$) por la metformina. Además, se detectó una correlación significativa entre iNOS y AMPK, p53, así como biomarcadores de pancreatitis aguda (amilasa, lactato deshidrogenasa y mieloperoxidasa). Por lo tanto, estos hallazgos demuestran una asociación entre la pancreatitis aguda y la modulación del eje pancreático AMPK-iNOS-p53 mientras está protegido por metformina.

PALABRAS CLAVE: Pancreatitis aguda; iNOS; p53; AMPK; Metformina, Modelo de rata.

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